

AD \_\_\_\_\_

Award Number: DAMD17-03-1-0399

TITLE: Migratory Leukocytes in the Pathogenesis and Diagnosis of  
Prion Disease

PRINCIPAL INVESTIGATOR: Alan J. Young, Ph.D.

CONTRACTING ORGANIZATION: South Dakota State University  
Brookings, South Dakota 57007-0497

REPORT DATE: June 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

**BEST AVAILABLE COPY**

20040922 037

**REPORT DOCUMENTATION PAGE**Form Approved  
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

<b>1. AGENCY USE ONLY</b> (Leave blank)		<b>2. REPORT DATE</b> June 2004	<b>3. REPORT TYPE AND DATES COVERED</b> Annual (15 May 2003 - 14 May 2004)	
<b>4. TITLE AND SUBTITLE</b> Migratory Leukocytes in the Pathogenesis and Diagnosis of Prion Disease			<b>5. FUNDING NUMBERS</b> DAMD17-03-1-0399	
<b>6. AUTHOR(S)</b> Alan J. Young, Ph.D.				
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> South Dakota State University Brookings, South Dakota 57007-0497  <i>E-Mail:</i> Alan.young.sdstate.edu			<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			<b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>	
<b>11. SUPPLEMENTARY NOTES</b>				
<b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited				<b>12b. DISTRIBUTION CODE</b>
<b>13. ABSTRACT (Maximum 200 Words)</b> Of all infectious diseases, prions remain the least understood and most difficult to study. Although it is generally accepted that immune cells are key components to the pathogenesis of the disease, relatively little is understood regarding their involvement. It is the goal of this work to understand the role(s) of migratory immune cells, including B cells and dendritic cells, in the pathogenesis of transmissible spongiform encephalopathies. To this end, we have two specific tasks: to understand the expression of the normal prion protein on immune cells in sheep, and to use that knowledge to investigate the potential role of migratory B cells in TSEs. Here, we describe our work which clearly demonstrated differences in the expression of PrP on the surface of B cells and B cell subsets, linked to the genetic background of the animal (i.e. scrapie susceptibility). In addition experiments on scrapie infected animals, we found that these same subsets appeared to be disrupted in infected as compared to normal animals, suggesting a potential effect of scrapie on the differentiation of B cells in lymph node germinal centers. This work has direct relevance to both the understanding and potential diagnosis of prion diseases in sheep and other species.				
<b>14. SUBJECT TERMS</b> B cells, dendritic cells, PrPSc, Diagnosis				<b>15. NUMBER OF PAGES</b> 12
				<b>16. PRICE CODE</b>
<b>17. SECURITY CLASSIFICATION OF REPORT</b> Unclassified	<b>18. SECURITY CLASSIFICATION OF THIS PAGE</b> Unclassified	<b>19. SECURITY CLASSIFICATION OF ABSTRACT</b> Unclassified	<b>20. LIMITATION OF ABSTRACT</b> Unlimited	

## Table of Contents

Cover.....	1
SF 298.....	2
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	11
Reportable Outcomes.....	11
Conclusions.....	12
References.....	12
Appendices.....	12

**Introduction:**

Although there is currently no evidence to support an active role for the immune system in defense against prion disease, cells of the immune system appear to play a critical role in the pathogenesis of known transmissible spongiform encephalopathies. The purpose of our work is to examine the role of migratory cells in the development of known prion diseases, including scrapie and chronic wasting disease. Using existing reagents, we have examined both the normal and pathogenic form of prion protein on cells of the immune system in normal and infected animals, in order to better understand the interaction of immune cells in disease development. Of all prion diseases, scrapie remains the best characterized, and we have chosen to examine differences in populations of B cells, follicular dendritic cells, and dendritic cells over the course of prion disease. We have previously identified a second subset of B cells which first becomes pronounced in ruminants at sexual maturity, and continues to grow in size throughout adulthood (Young et al., 1997). As a result, we have chosen to focus on this subset as a potential target to better understand disease pathogenesis and potentially develop more sensitive antemortem assays for diagnosis of prion disease. To this end, we have focused our initial work on defining these subsets in experimentally infected sheep. While we have chosen to focus on an ovine model, we believe that our results will be generally applicable to a greater understanding of prion diseases in other species as well.

**Report Body**

Our efforts to better understand immune involvement in prion disease can be broadly broken down into two distinct tasks:

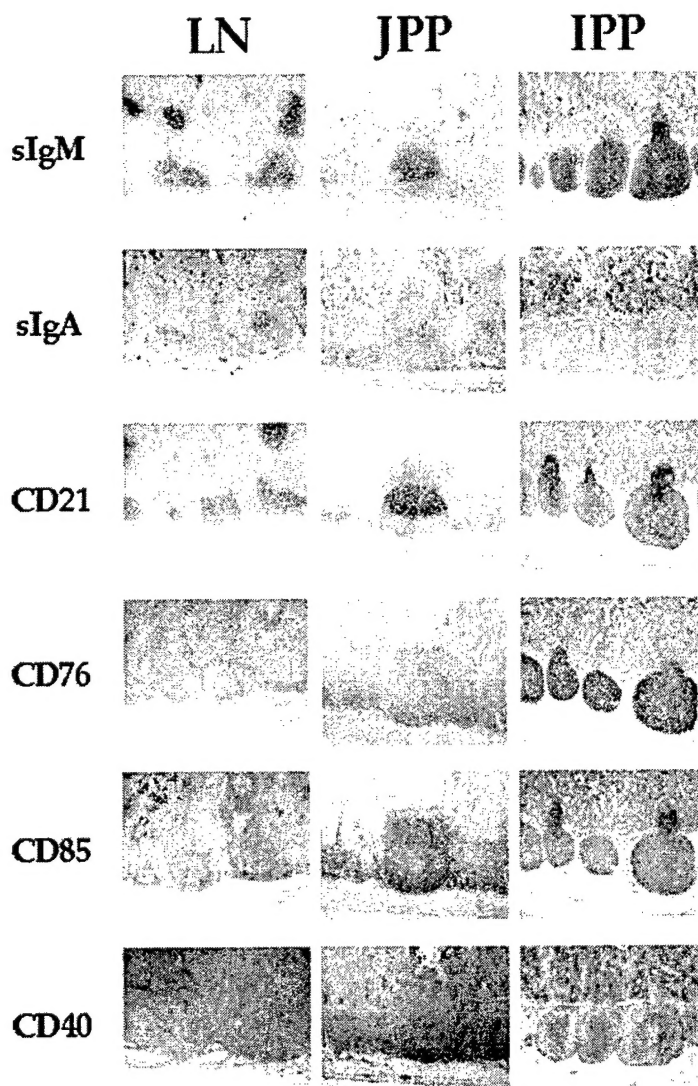
1. To define the expression of cellular prion protein on previously-established B and dendritic cell subsets
2. To define the expression of *pathogenic* prion protein on the surface of migratory leukocytes from infected animals.

Although these two aims are intrinsically intertwined in our work, we will describe our progress towards these two tasks separately.

1. Expression of cellular prion protein.

In order to better understand the normal function of prion protein (PrP<sup>c</sup>) in immune cells, we have used flow cytometry to characterize the expression of PrP on the surface of B cells and Follicular Dendritic cells (FDCs) during development and immune function. Ruminants and some other domestic animals are unique from mice, in that B cells undergo an obligatory stage of development within a specialized, gut-associated lymphoid organ grossly similar in function to the Bursa of Fabricius in chickens. Very briefly, the ileal Peyer's patch (IPP) is seeded by a small number of functionally immature B cells during embryonic development. While these B cell precursors have fully-rearranged immunoglobulin genes, they are unable to respond to antigen in a

manner similar to mature B cells, and additional diversification is added in the IPP through extensive cell division and antigen-independent somatic hypermutation. It is intriguing that the IPP has also been described as the earliest organ to contain histologically identifiable PrPSc following oral infection (Heggebo et al., 2000). In an effort to investigate potential differences in the B cell populations in the IPP as compared to secondary lymphoid organs such as the Jejunal Peyer's patches and retropharyngeal lymph node, we used a panel of antibodies to define B cell populations unique to each tissue. To this end, we stained cell suspensions and immunohistochemical sections with antibodies specific for B cell surface molecules including CD40, CD11b, CD11c, CD21, CD35, CD85, CD72, CD75s, CD62L, and surface immunoglobulin (Figure 1). Intriguingly, a highly effective marker for identifying various stages of B cell development was CD21. This is especially significant, as CD21 is a receptor for complement component C3, which has been shown to be involved in prion pathogenesis in murine models (Klein et al., 1997; McBride et al., 1992).

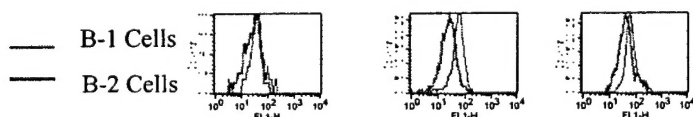


**Figure 1: Expression of cell surface molecules on B cells in secondary lymphoid tissues (Retropharyngeal Lymph Node, Jejunal Peyer's patch) and primary lymphoid tissues (Ileal Peyer's patch).**

Specifically, CD21 is regulated with B cell development such that immature B cells express very low levels, which increase as the B cell matures. This expression appears to remain until activation within B cell follicles in lymph nodes, at which point expression is lost at least temporarily. Intriguingly, we found that expression of PrP<sup>c</sup> on the surface of B cells was inversely regulated with CD21 expression in both the IPP and secondary lymphoid tissues. We are currently examining the significance of this result.

In additional studies, we examined the expression of PrP<sup>c</sup> on the surface of B cell subsets found in the peripheral blood. We have previously defined two major subsets of peripheral blood B cells, which can be differentiated based upon the expression of complement receptors. Conventional, B-2 subsets can be identified based upon their expression of CD21 and failure to express CD11b and CD11c, whereas so-called B-1 cells express high levels of CD11b and CD11c but fail to express CD21. Work from our laboratory has demonstrated that B-1 cells appear to be derived from a B-2 precursor, as a result of differentiation within lymph nodes. In order to define any potential differences in PrP<sup>c</sup> expression on the surface of circulating B-1 and B-2 subsets, we collected blood from sheep of known PrP genotype (so-called QQ, QR, and RR animals) and performed semi-quantitative flow cytometry using mAb 6H4 to define any differences in cell-surface expression of prion protein. As a result of these studies, we made two surprising observations (Figure 2). Firstly, there appears to be a significant difference in the expression of prion protein between these three genotypes, such that QQ and QR sheep express significantly more surface PrP on B cells than RR animals ( $P < 0.05$ ). In addition, CD21 negative B-1 cells also express significantly less prion protein than CD21+ B cells, regardless of genotype ( $p < 0.05$ ). These data suggest significant differences in expression of prion protein on B cells that may correlate with genotype, and may play a role in disease susceptibility. Furthermore, these differences in PrP expression between CD21+ and CD21- mature B cell subsets confirms the above expression data on developing B cell subsets. We are currently banking samples to confirm these results through quantitative real-time RT-PCR.

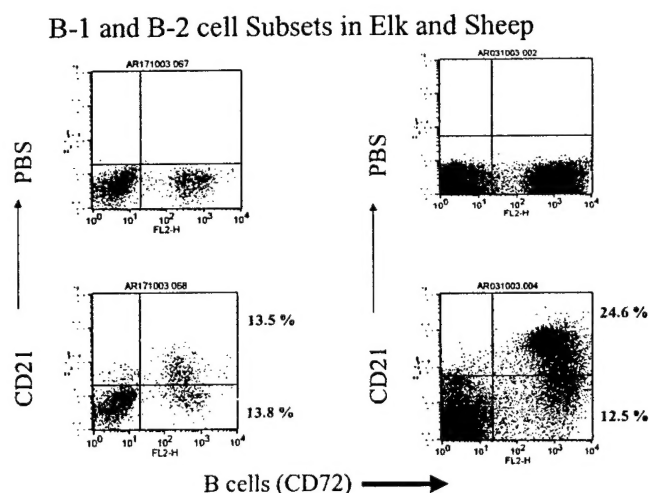
### Ovine B-2 Cells Express Higher Levels of Native PrP<sup>c</sup> Than B-1 Cells



	RR Genotype	QR Genotype	QQ Genotype
B-1 Cell Mean Intensity	26.1 ± 3.6	36.3 ± 3.1	35.0 ± 5.9
B-2 Cell Mean Intensity	32.9 ± 5.4	52.3 ± 1.0	48.6 ± 9.2

**Figure 2: Expression of Prion protein on the surface of B cell subsets in genetically resistant and susceptible sheep.**

In addition to the above flow cytometric work, we have been characterizing antibody combinations which can be used for the identification of B cell subsets and follicular dendritic cell subsets in histological sections. While this work is ongoing, we have found a panel of 3 FDC-specific antibodies which appear to identify FDCs in formalin-fixed tissues. We are currently working to confirm these results. In related work, we have been working to define reagents which will allow us to confirm the generality of these results to other transmissible spongiform encephalopathies. To this end, we have tested reactivity of our B and FDC antibodies against cells from Deer and Elk. As a result, we have found a panel of 17 antibodies which successfully identify cells by flow cytometry, including several which segregate B-1 and B-2 cell populations in cervids (Figure 3). While this work is ongoing, at least 2 of these antibodies successfully identify similar cell populations in formalin-fixed lymph nodes from elk.



**Figure 3: Cervids (left panels) express both B-1 and B-2 Subsets in Blood, similar to sheep**

**Task 2: Definition of the expression of pathogenic prion protein on the surface of migratory leukocytes from infected animals.**

The ultimate goal of this task is to define cells of the immune system carrying or affected by prion infection. To this end, we have collaborated with scientists at the National Animal Disease Center, Ames, IA to examine migratory cell subsets in experimentally infected animals. To date, we have collected blood samples from a total of 20 animals at various times after either intracerebral (10) or oral (10 animals) infection. Of these, 10 animals were genetically resistant to disease (so-called "QR") animals and 5 animals infected intracerebrally with scrapie brain homogenate and 1 animal infected orally have succumbed to disease. Samples of blood are collected bi-monthly, and samples of blood, retropharyngeal lymph node, mesenteric lymph node and brain are collected at necropsy. As we have directed our efforts towards isolating migratory B cell and dendritic cell subsets which are potentially involved in disease pathogenesis, we have confined our

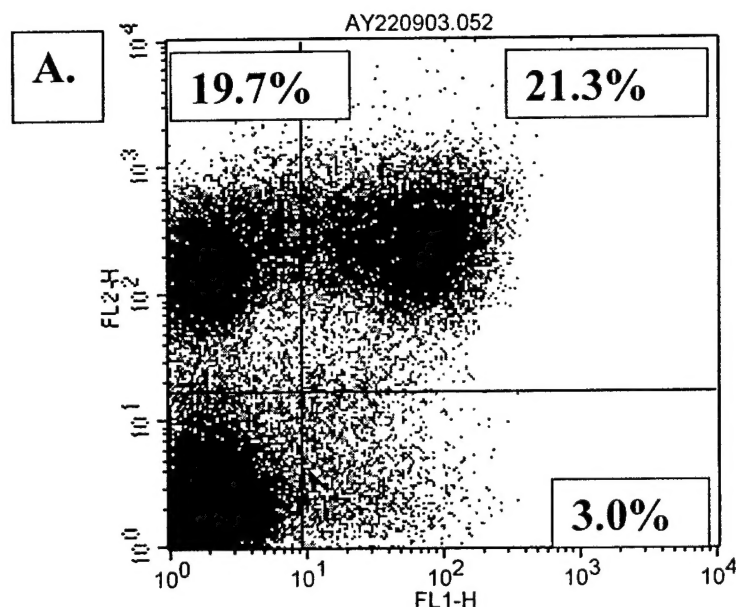


analysis to these two populations. To this end, we have developed methods to examine differences in B cell subpopulations in the blood of scrapie infected animals, and methods to separate both B and FDC populations from the blood and lymph nodes of scrapie-positive animals. Although this work is ongoing, several observations have been worthy of note and further investigation. Firstly, we have used a sensitive western-blot technique to test for the presence of scrapie protein in lymphoid populations separated using a multi-step magnetic bead separation protocol. Using this technique, scrapie protein could be reliably identified in CD21+ but not CD21-ve cells isolated from retropharyngeal lymph nodes of infected animals.

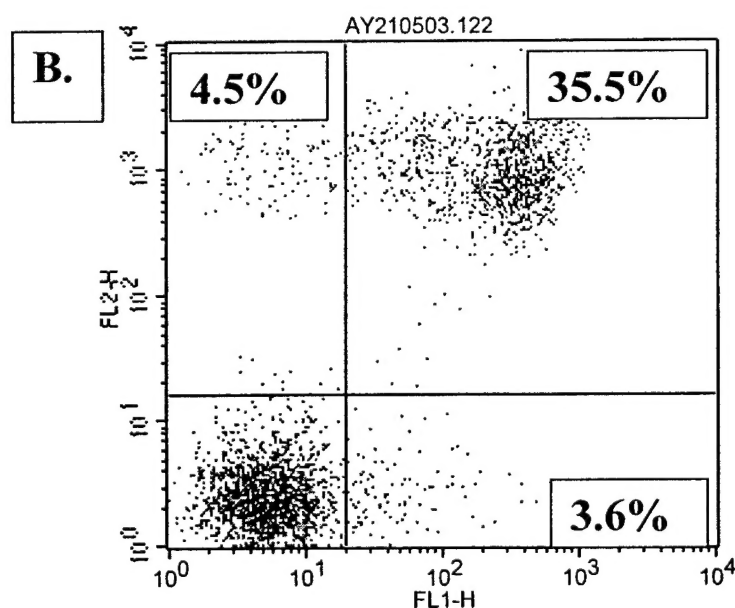
Analysis of peripheral blood populations has proven to be even more interesting. As a result of the analysis described above, we have found that animals infected with scrapie (and eventually succumbing to disease) possess a significantly elevated CD11c+ B cell population in the peripheral blood. While this is not reflected in higher *numbers* of these cells, the *proportion* of CD11c+ B cells in the blood of these animals appears elevated relative to controls (Figure 4). While significant, it should be mentioned that elevated numbers of these cells could also be detected in animals undergoing significant inflammation: one animal which did not succumb to scrapie but had elevated CD11c+ B cell numbers died of abdominal ulcers, and a second had a significant intradermal granuloma unrelated to the study. Nonetheless, these promising results have led us to further investigate the means whereby CD11c+ B-1 cells are generated within the germinal centers of lymph nodes. Given that FDCs have been found to be crucial to normal infection with scrapie and are routinely identified as containing PrPSc in even intracerebrally infected animals, we believe that these cells are central to understanding the pathogenesis of prion diseases and form the focus of our efforts devoted to understanding the early stages of scrapie infection (currently underway).

In order to fully explore the role of Follicular Dendritic Cells in prion pathogenesis, we are attempting to answer 2 questions: How is PrPSc transported from the initial site of infection (the gut) to the follicular dendritic cells of regional lymph nodes, and how does the "infection" of FDCs affect the differentiation of ovine B-1 and B-2 cells? As described in our proposal, we intend to use a method of direct lymphatic cannulation to track prion transport from the periphery to regional lymph nodes and ultimately to the follicular dendritic cells. We have ordered genetically defined scrapie susceptible animals for these experiments, and will be performing these experiments later this year. In preparation, we have been using multi-colour flow cytometry to define dendritic cell subsets in afferent lymph. Similar to previous reports, we have found that antibodies specific for CD205 (DEC205) reliably identify all DCs in afferent lymph, which can then be differentiated into two subpopulations based on expression of CD11c. In addition, we have also found a small subpopulation of CD11c+ DCs which express a surface antigen recognized by one of our FDC-specific antibodies. In related studies, we have developed a surgical procedure which can be used to collect afferent lymph directly draining the sheep brain, which will be used in future studies to map the response of the brain to scrapie infection.





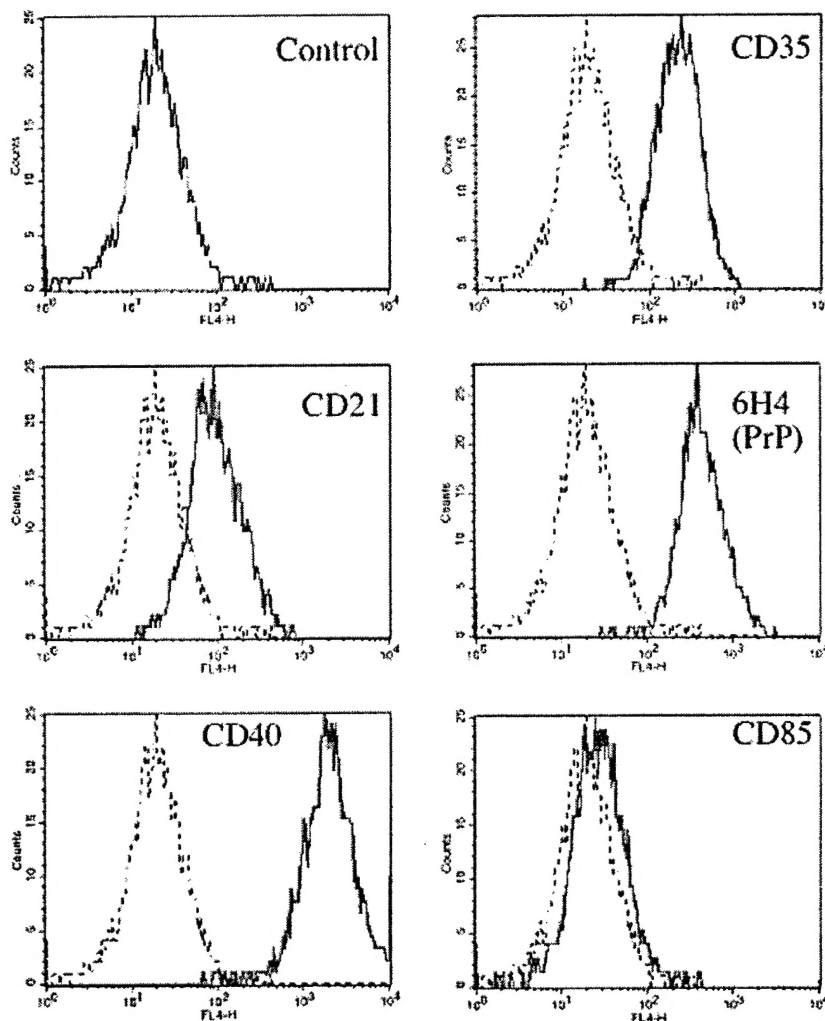
B-2 Cells	B-1 Cells



**Figure 4: Disproportionate Representation of B-1 cells in Scrapie Infected Sheep.** Panel A shows the normal, equal representation of B-2 and B-1 cells (upper Left and Upper Right Quadrants) in a non-infected sheep. Panel B shows the peripheral blood B cell profile of an animal infected 18-months previously with scrapie-infected brain. Although this animal was asymptomatic, pathogenic PrP<sup>Sc</sup> was demonstrated post-mortem.

In order to develop an in-vitro model to examine the interaction of prion protein with FDCs and B cell subsets, we have successfully developed primary FDC cell lines from both scrapie positive and negative animals. Briefly, FDC-specific antibodies were used to magnetically separate cells, which were then cultured in rich tissue culture media.

These large cells were morphologically similar to FDCs, and expressed CD21, CD40, CD35, and several other surface markers consistent with FDCs (Figure 5). In initial studies, these cells were also capable of inducing B cell proliferation, demonstrating that we have successfully developed ovine FDC cell lines which can be used for further study of prion pathogenesis in vitro.



**Figure 5: Expression of FDC-Associated antigens on the surface of cultured FDC cell lines (solid lines) relative to control staining (dotted line).**

In summary, we have performed all baseline data necessary to successfully perform acute studies on scrapie infectivity. We have defined an interesting link between CD21 expression on B cells and scrapie infectivity, as well as defining a potentially critical alteration in the generation of B-cell subpopulations in scrapie infected animals. In control experiments, we have defined populations of migrating DCs of interest in afferent lymph, and developed the means to track the transport of PrPSc from the gut or the brain to regional lymph nodes. Our successful development of ovine FDC cell lines will further allow us to model the interaction of PrPSc with FDCs and other cell types normally found within the germinal center.

**Key Research Accomplishments:****Task #1: Normal PrP Expression,**

- Definition of the Differential Expression of PrPC on B cells of QQ, QR and RR animals.
- Definition of the Differential Expression of PrPC on ovine B-1 and B-2 cells in peripheral blood.
- Definition of the inverse linkage between PrPC and CD21 expression on ovine B-cell subsets from ileal Peyer's patches, jejunal Peyer's patches, and lymph nodes.
- Identification of reagents which can be used to define B-1 and B-2 subsets in deer and elk.
- Identification of antibodies which can be used to identify B-1, B-2, FDC, and DC subsets by immunohistochemistry.

**Task#2**

- Banking of cell suspensions from scrapie positive and control animals infected either intracerebrally or orally.
- Validation of a magnetic cell-sorting procedure to define PrPSc infection of B, FDC, and DC subsets by western blot.
- Definition of a unique shift in the proportion of B-1 and B-2 lymphocytes in scrapie infected animals as compared to age-matched controls.
- Culture of ovine FDCs from normal and scrapie-infected animals.
- Characterization of DC subpopulations found in normal afferent lymph.

**Reportable Outcomes:****1) Abstracts and Presentations:**

Elmubark, G.E., Graybill, M., DeRycke, M., Young, A.J. Migration and Differentiation of Afferent Lymph Dendritic Cells as a Model of APC Function in Sheep. Presented at the 2003 Autumn Immunology Conference, Chicago IL. November 2003.

Rohlik, A., DeRycke, M., Young, A.J. Identification and Characterization of Leukocyte Populations in Hoofstock; A Tool for the Study of Chronic Wasting Disease (CWD). Presented at the 2003 Autumn Immunology Conference, Chicago IL. November 2003.

## 2) Cell Lines Developed:

-Several FDC cell lines from normal and infected sheep.

## 3) Additional Grant Applications Submitted based on this Work:

STTR Submitted in collaboration with Rural Technologies Inc. in response to ARO STTR topic A04-027, Development of Cell Lines from Deer, Elk, and Humans for use in development of Assays for Prion Diseases.

**Conclusions:**

The unique linkages between PrPC expression and B cells have been further confirmed through identification of distinct differences of PrPC expression on developing B cell subsets. The fact that these differences appear to correlate with expression of CD21 further indicate a potential role between complement and prion infection. Our identification of apparent differences in the proportions of B cell subsets in normal and scrapie infected animals suggests a potential effect on the immune system of prion infection. While the most interesting application of this knowledge is towards a greater understanding pathogenesis, the potential use of this information for further diagnostic assays cannot be discounted. Our development of FDC-cell lines which can potentially be used to isolate any affect of PrPSc on normal germinal center function, as well as a potential tool to propagate prions in vitro will be a useful tool in further research.

**References:**

- Heggebo, R., Press, C.M., Gunnes, G., Lie, K.I., Tranulis, M.A., Ulvund, M., Groschup, M.H., Landsverk, T. 2000. Distribution of prion protein in the ileal Peyer's patch of scrapie-free lambs and lambs naturally and experimentally exposed to the scrapie agent. *J. General Virol.* 81(Pt9):2327-2337.
- Klein, M.A., Frigg, R., Flechsig, E., Raeber, A.J., Kalinke, U., Bluethmann, H., Bootz, F., Suter, M., Zingernagel, R.M., Aguzzi, A. 1997. A crucial role for B cells in neuroinvasive scrapie. *Nature* 390:687-691
- McBride, P.A., Eikelenboom, P., Kraal, G., Fraser, H., Bruce, M.E. 1992. PrP protein is associated with follicular dendritic cells of spleens and lymph nodes in uninfected and scrapie-infected mice. *J. Pathol.* 168:413-41
- Young, A.J., Marston, W.L., Dessing, M., Dudler, L. Hein, W.R. 1997. Distinct recirculating and non-recirculating B lymphocyte pools in the peripheral blood are defined by coordinated expression of CD21 and L-selectin. *Blood* 90:4865-4875.

**Appendices**

None